Drosophila Glypicans Regulate Follicle Stem Cell Maintenance and Niche Competition

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ABSTRACT Adult stem cells reside in specialized microenvironments called niches, which provide signals for stem cells to maintain their undifferentiated and self-renewing state. To maintain stem cell quality, several types of stem cells are known to be regularly replaced by progenitor cells through niche competition. However, the cellular and molecular bases for stem cell competition for niche occupancy are largely unknown. Here, we show that two *Drosophila* members of the glypican family of heparan sulfate proteoglycans (HSPGs), Dally and Dally-like (Dlp), differentially regulate follicle stem cell (FSC) maintenance and competitiveness for niche occupancy. Lineage analyses of glypican mutant FSC clones showed that *dally* is essential for normal FSC maintenance. In contrast, *dlp* is a hypercompetitive mutation: *dlp* mutant FSC progenitors often eventually occupy the entire epithelial sheet. RNA interference knockdown experiments showed that Dally and Dlp play both partially redundant and distinct roles in regulating Jak/Stat, Wg, and Hh signaling in FSCs. The *Drosophila* FSC system offers a powerful genetic model to study the mechanisms by which HSPGs exert specific functions in stem cell replacement and competition.

KEYWORDS heparan sulfate; glypicans; stem cell competition; follicle stem cells; *Drosophila*

ALTHOUGH stem cell activity is maintained throughout adult life, individual stem cells often have limited lifespans (Margolis and Spradling 1995). Adult stem cells are replaced regularly to ensure that the niche is always inhabited with functional stem cells (Xie and Spradling 2000; Ryu *et al.* 2003; Nystul and Spradling 2007). One mechanism of stem cell replacement is competition for niche occupancy between stem cells and their replacement-competent daughters (Nystul and Spradling 2007, 2010; Jin *et al.* 2008). However, the cellular and molecular bases for stem cell competition for niche occupancy are largely unknown.

Drosophila ovarian follicle stem cells (FSCs) offer an excellent model to study stem cell behavior in an epithelial tissue (Sahai-Hernandez *et al.* 2012). The *Drosophila* ovary is composed of 16–20 parallel tubes called ovarioles that contain developing egg chambers arranged in a linear array of progressive developmental stages. During oogenesis, the developing oocyte is interconnected with 15 sister cells, called nurse cells. These developing germ cells are surrounded and supported by a somatic epithelium composed of several different types of somatic follicle cells, which undergo multiple rounds of reorganization to determine the shape of the egg. All follicle cells in each ovariole are derived from two FSCs that reside in separate niches, one on each side of the germarium, which is the most anterior structure of the ovariole (Margolis and Spradling 1995). These FSCs are replaced by a daughter of the remaining stem cell; FSC daughters regularly migrate across the germarium and compete with resident stem cells for niche occupancy (Nystul and Spradling 2007, 2010). Although first identified in *Drosophila*, this stem cell behavior appears to be a general characteristic of stem cells, including in mammals (Li and Clevers 2010). Since abnormally competitive behaviors of stem cells resemble precancerous events in human epithelia, FSC maintenance and niche competition provide a powerful model to investigate epithelial cancer formation (Nystul and Spradling 2007).

We have previously demonstrated that heparan sulfate proteoglycans (HSPGs) are essential regulators of *Drosophila* adult stem cells, including germline stem cells (GSC) in the ovary (Hayashi *et al.* 2009; Dejima *et al.* 2011) and the testis (Levings *et al.* 2016), and the intestinal stem cells in the

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midgut (Takemura and Nakato 2017). HSPGs are a class of carbohydrate-modified proteins composed of heparan sulfate (HS) chains, a long, unbranched glycosaminoglycan, covalently linked to a core protein. They play key roles in numerous biological processes such as growth factor signaling, cell adhesion, and enzymatic catalysis (Esko and Selleck 2002; Kirkpatrick and Selleck 2007). As one of their most important functions, HSPGs serve as coreceptors for secreted signaling ligands. Such HS-dependent factors include fibroblast growth factors, bone morphogenetic proteins, Wnt/Wingless (Wg), Hedgehog (Hh), and Unpaired (Upd), a ligand of the *Drosophila* Jak/Stat pathway (Li and Kusche-Gullberg 2016; Nakato and Li 2016). HSPGs regulate both signal reception on the cell surface and distribution of the ligand proteins in a tissue (Fujise *et al.* 2003).

Previous studies of the FSC niche have identified several signaling pathways essential for FSC maintenance. For example, the Hh and Jak/Stat pathways were shown to be key regulators for FSC maintenance (Forbes *et al.* 1996; Zhang and Kalderon 2000; Hartman *et al.* 2010; Vied *et al.* 2012). In addition, Wg signaling plays a critical role for FSC maintenance (Song and Xie 2003; Sahai-Hernandez and Nystul 2013). FSC behavior in response to these signals is dosage-dependent, and reception of signaling ligands at FSCs must be tightly regulated (Vied *et al.* 2012). However, how these pathways are integrated and orchestrated to regulate FSC maintenance and replacement remains to be elucidated. All the ligands of these three pathways, Upd, Hh, and Wg, are known to be HS-dependent and require HSPG coreceptors for proper signaling. This raises the possibility that HSPGs affect multiple signaling pathways to coordinate FSC behavior.

In this study, we asked whether glypicans affect behaviors of FSCs, namely their turnover and replacement. We found that both Dally and Dally-like (Dlp) are expressed in FSCs and regulate FSC maintenance and competition. Interestingly, *dally* and *dlp* mutant FSC clones showed opposite behaviors: *dally* mutant FSCs are less competitive for niche occupancy compared to wild type, while *dlp* acts as a hypercompetitive mutation. *dlp* mutant FSC progenitors often eventually occupy the entire epithelial sheet. This phenomenon resembles an early phase of cancer formation. These findings are consistent with previous observations that some human glypicans are oncogenic and others are tumor suppressors. We show that the two glypicans play both partially redundant and distinct roles in regulating Jak/Stat, Wg, and Hh signaling in FSCs.

Materials and Methods

Fly strains

Detailed information for the fly strains used are described in FlyBase (http://flybase.bio.indiana.edu/) except where noted. The wild-type strains used were Canton-S or w^{1118}, a white eye strain backcrossed 20 times to Canton-S. Other strains used were as follows: $dally^{CPTI001339}$, a YFP protein trap line inserted in the endogenous *dally* locus (Lowe *et al.* 2014); $dlp^{CPTI000445}$, a GFP protein trap line inserted in the endogenous *dlp* locus (Lowe *et al.* 2014); $Sdc^{CC00871}$, a GFP protein trap line inserted in the endogenous *Sdc* locus (Buszczak *et al.* 2007), $robo1^{HA\text{-}robo1}$, $robo2^{HA\text{-}robo2}$, and $robo3^{HA\text{-}robo3}$, endogenously HA-tagged strains for *robo1*, *robo2*, and *robo3*, respectively (Spitzweck *et al.* 2010); sfl^{9B4}, a null allele of *sulfateless* (*sfl*) (Lin and Perrimon 1999); $dally^{gem}$, a null allele of *dally* (Tsuda *et al.* 1999); $dally^{80}$, a null allele of *dally* (Han *et al.* 2004); $dally^{MH32}$, a null allele of *dally* (Franch-Marro *et al.* 2005); dlp^{A187}, a null allele of *dlp* (Han *et al.* 2004); dlp^{MH20}, a null allele of *dlp* (Franch-Marro *et al.* 2005); *109-30-Gal4* (Bach *et al.* 2007; Hartman *et al.* 2015); *UAS-dally* (Takeo *et al.* 2005); *UAS-dlp* (Kleinschmit *et al.* 2010); *UAS-sfl* $RNAi^{HMS00543}$, an upstream activating sequence (UAS) short-hairpin RNA interference (RNAi) strain for *sfl* (Levings *et al.* 2016); *UAS-dally* $RNAi^{GD5918}$, a UAS short-hairpin RNAi strain for *dally* (Dietzl *et al.* 2007); *UAS-dlp* $RNAi^{HMS00875}$, a UAS short-hairpin RNAi strain for *dlp* (Ni *et al.* 2011); *UAS-Sdc* $RNAi^{GD4545}$, a UAS short-hairpin RNAi strain for *Sdc* (Dietzl *et al.* 2007); *10xSTAT-GFP*, a reporter strain for monitoring Jak/Stat signaling (Vied *et al.* 2012); *frizzled 3* (*fz3*)*-RFP*, a reporter strain for monitoring Wg signaling (Wang and Page-McCaw 2014); and *ptc-GFP*, a reporter strain for monitoring Hh signaling (Ulmschneider *et al.* 2016).

FSC maintenance assay

FSC maintenance and competition behavior were examined by mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo 2001). This method measures the persistence of marked FSC lineages of a defined genotype that are generated by heat shock–induced, FRT-mediated mitotic recombination (Nystul and Spradling 2007). When mitotic clones are generated at low frequency, most of the germaria have either zero or one FSC labeled. Over time, interniche FSC replacement occurs, causing a decrease in the frequency of germaria with one labeled FSC. Any reduction or increase in the frequency of marked mutant FSC clones relative to marked control clones at 7 days or thereafter reflect a selective loss or gain of FSC clones, respectively, that could be attributed to their mutant genotypes.

To induce MARCM clones, *y w hs-FLP tub-Gal4 UAS-GFP* [*nls*];; *FRT2A tub-*Gal^{80} was crossed to wild-type or mutant chromosomes with FRT2A to obtain *y w hs-FLP tub-Gal4 UAS-GFP*[*nls*]/+;; *FRT2A tub-Gal80/FRT2A* * (* represents respective mutations). Adult female flies that were 2–4 days old after eclosion were heat shocked twice for 1 hr, ~8 hr apart, for 2 days (total four heat shock treatments) at 37° and then were kept at 25° for 7–21 days before dissection. FSC lineages were examined at 7, 14, and 21 days post heat shock (dphs).

FSC replacement bias assay

FSC mutant clones were induced in the same manner as the FSC maintenance assay. The percentages of germaria with zero, one, or two marked FSCs were measured at 7 and 21 dphs. The increase in zero or two marked FSC clones

between 7 and 21 dphs were analyzed to determine if the marked cells have a niche competition advantage or disadvantage compared to control. The ratio between these two events was calculated.

RNAi knockdown

A Gal4 driver strain was crossed to *UAS-sfl RNAi*, *UAS-dally RNAi*, or *UAS-dlp RNAi* strain at 25°. For control animals, the Gal4 driver was crossed to w^{1118}, which has the same genetic background as the UAS-RNAi lines used. Adult female flies bearing both the Gal4 driver and UAS-RNAi transgene were transferred to a new vial at 0–2 days after eclosion. They were cultured with males at 29° for additional 7 days and dissected on day 7 post temperature shift. The flies were transferred to fresh food at least once every 2 days. *109-30-Gal4* was used for gene knockdown in FSCs/follicle cells.

Signaling reporter assays

To monitor Jak/Stat, Wg, and Hh signaling in the FSCs, we used the following transgenic reporter constructs: 10xSTAT-GFP for Jak/Stat signaling (Vied *et al.* 2012), fz3-RFP for Wg signaling (Wang and Page-McCaw 2014), and ptc-GFP for Hh signaling (Ulmschneider *et al.* 2016). To quantify GFP or RFP signals of reporter assay experiments, GFP or RFP signal intensity was measured over the area of FSCs. FSCs were identified by their location at the 2a/2b border, triangular shape, and low *Fas*III staining. We used the polygon selection tool in ImageJ to select the FSC area, followed by the Measure function. The same setting was used to acquire images within each signaling pathway (set of experiments) to capture the differences in GFP or RFP intensities. We then compared the GFP or RFP intensity values in FSC regions between control and individual RNAi knockdown samples. The intensity values in control samples were set to 1.0, and relative intensities in RNAi knockdown FSCs were calculated. Student's *t*-test was used for statistical analysis.

Stalk cell quantification

We counted the number of the first stalk cell (after region 3 of the germarium) and the second stalk cell (after stage 2 egg chamber) of control and RNAi knockdown samples. The number of stalk cells was calculated from three independent experiments. A Wilcoxon signed rank test was used as a statistical analysis between control and RNAi knockdown samples.

Immunohistochemistry and microscopy

Immunostaining was performed as previously described (Fujise *et al.* 2001; Hayashi *et al.* 2009, 2012). Briefly, samples were fixed for 15 min with 4% formaldehyde in PBS and washed with 0.1% PBS Triton X-100 (PBST) (20 min, three times). They were then blocked in 2% normal goat serum in 0.1% PBST for 30 min and incubated overnight in primary antibodies at 4°. Samples were again washed in 0.1% PBST (20 min, three times) and incubated with the appropriate Alexa Fluor secondary antibodies overnight at 4°. They were then washed in 0.1% PBST (20 min, three times), before being mounted in VECTASHIELD (H-1000 or H-1200; Vector Laboratories, Burlingame, CA). Images were obtained using either a Zeiss 710 or a Nikon Eclipse E800 laser scanning confocal microscope.

The following primary antibodies were used: mouse anti-*Fas*III [1:100; Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-GFP (1:1000; Invitrogen, Carlsbad, CA), rat anti-Vasa (1:100; DSHB), and mouse anti-Dlp (1:50; DSHB). Secondary antibodies conjugated with Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 633 were used in 1:500 dilutions (Molecular Probes, Eugene, OR).

Data availability

The authors affirm that all data necessary for confirming the conclusions of this article are represented fully within the article and its tables and figures. Supplemental figures show the role of Syndecans (Sdcs) in the FSCs (Supplemental Material, Figure S1 in File S1) and expression patterns of the Robo receptors in the germarium (Figure S2 in File S1). Fly strains and reagents are available upon request.

Results

Expression patterns of dally and dlp in the developing ovary

As the first step in analyzing glypican functions in the developing ovary, we determined the expression patterns of Dally and Dlp using protein trap lines, $dally^{CPTI001339}$ and $dlp^{CPTI000445}$. These lines bear a GFP or YFP insertion in frame in an intron of their respective endogenous genes (Morin *et al.* 2001; Buszczak *et al.* 2007; Quinones-Coello *et al.* 2007; Lowe *et al.* 2014). Animals homozygous for these GFP/YFP fusion genes do not show any phenotypes, indicating that the insertions do not disrupt gene functions and protein localization. Therefore, the GFP/YFP distribution reflects the localization of native proteins. Dally-YFP in $dally^{CPTI001339}$ is expressed exclusively in somatic cell populations. In addition to its expression in the cap cells as previously reported (Hayashi *et al.* 2009), Dally-YFP is detectable in the posterior part of the germarium: it is expressed in the most posterior row of escort cells and in the follicle cell populations, including the FSCs and differentiating follicle cells (Figure 1, B and B'). Dally-YFP is not expressed in the anterior part of the escort cells or germline cells. Previous studies have shown that Dally on the surface of the cap cells acts as a *trans*-coreceptor to mediate Dpp signaling only in the contacting GSCs (Guo and Wang 2009; Hayashi *et al.* 2009; Liu *et al.* 2010). Therefore, the absence of Dally in anterior escort cells is important to maintain a normal number of GSCs.

Upd, a ligand of the *Drosophila* Jak/Stat pathway is known to form a concentration gradient on the apical surface of follicle cells in the developing egg chamber and acts as a morphogen to regulate follicle cell differentiation (Xi *et al.* 2003; Hayashi *et al.* 2012). We have previously shown that Dally serves as a coreceptor for Upd during this process

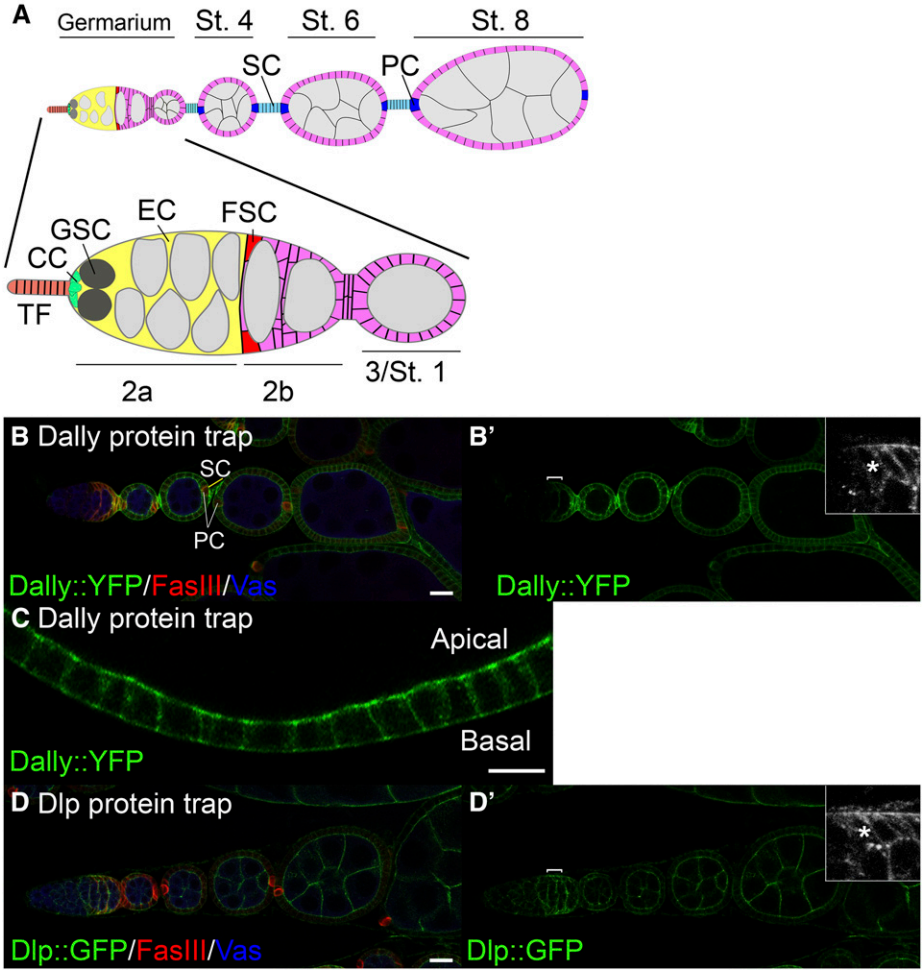

Figure 1 Expression patterns of Dally and Dlp in the ovary. (A) The anatomy of the *Drosophila* ovariole. The *Drosophila* ovary is composed of 16–20 ovarioles that contain progressively developing egg chambers. At the anterior tip of each ovariole, a structure called the germarium contains the two populations of stem cells, germline stem cells (GSC, dark gray) and follicle stem cells (FSC, red). There are two FSCs in each germarium and they reside in separate niches, one on each side of the germarium at the border of regions 2a and 2b. There are multiple somatic cell types in the germarium: terminal filament cells (TF, orange), cap cells (CC, niche cells for the GSCs, green), germ cells (light gray), follicle cells (pink), polar cells (PC) (blue), and escort cells (EC, light brown). (B and B') Expression of *dally* protein trap line. Anti-GFP antibody staining of $dally^{CPTI001339}$ ovary shows Dally-YFP expression in posterior escort cells in region 2a, FSCs, and follicle cells. Higher levels of expression are observed in polar cells (PC) (the Upd-expressing cells) and stalk cells (SC) (the Upd-receiving cells, marked by yellow line). Ovarioles were stained for GFP (green), Vas (a germline cell marker, blue), and *Fas*III (a differentiated follicle cell marker, red). A magnified view of FSC region (marked by bracket) is shown in the inset (B'). Asterisk in the inset marks the FSC. (C) A high magnification view of the follicle cell sheet of a $dally^{CPTI001339}$ egg chamber. A higher level of Dally-YFP expression is detected in the apical membrane compared to the basolateral membrane. (D and D') Expression of *dlp* protein trap line. Anti-GFP antibody staining of $dlp^{CPTI000445}$ ovary shows Dlp-GFP expression in the same region as Dally in the germarium. However, expression level is decreased in somatic cells after stage 3–4. Instead, Dlp but not Dally is expressed in germline cells after stage 2. A magnified view of FSC region (marked by bracket) is shown in the inset (D'). Asterisk marks the FSC. Bar, 10 μm.

(Hayashi *et al.* 2012). Using this *dally* protein trap line, we detected higher levels of Dally-YFP on the apical surface compared to the basolateral membrane of these cells (Figure 1C). This is consistent with the notion that Dally regulates Upd gradient formation because Upd is known to form a concentration gradient on the apical membrane of the follicle epithelial sheet (Xi *et al.* 2003; Hayashi *et al.* 2012). Intriguingly, expression of Dally-YFP is stronger in polar and stalk cells, which receive high levels of Upd-Jak/Stat signaling, than in main body follicle cells. This raises an interesting possibility that *dally* expression is induced by Jak/Stat signaling, forming a positive feedback loop for this pathway.

We also analyzed the expression patterns of Dlp-GFP in $dlp^{CPTI000445}$, a Dlp protein trap line. Anti-GFP antibody staining showed that Dlp is more broadly expressed than Dally in the germarium, being found in some of the anterior escort cells. It is also expressed in FSCs and differentiating follicle cells (Figure 1, D and D'). However, Dlp expression in somatic cells significantly decreases after stage 3–4. Conversely, Dlp expression begins to be detectable in germline cells at stage 2.

Glypicans regulate FSC maintenance

Since FSCs express both glypicans, we examined the functions of *dally* and *dlp* in controlling FSC behaviors. FSC maintenance and competition for niche occupancy can be analyzed by MARCM (Lee and Luo 2001; Takemura and Nakato 2015, 2017). This method allows us to visualize marked FSC lineages of a defined genotype that are generated by heat shock–induced, FRT-mediated mitotic recombination (Figure 2A). To study FSC maintenance, we quantified the number of germaria containing GFP-positive FSC clones at 7, 14, and 21 dphs (Figure 2B). The change in the percentage of germaria containing GFP-positive cells over time shows the retention of FSC progenitors with different genotypes in the germarium. As expected, in wild-type controls, a slow decrease in marked FSC clone frequency was observed due to normal slow turnover (Figure 2B; Zhang and Kalderon 2000; Song and Xie 2003; Kirilly *et al.* 2005;

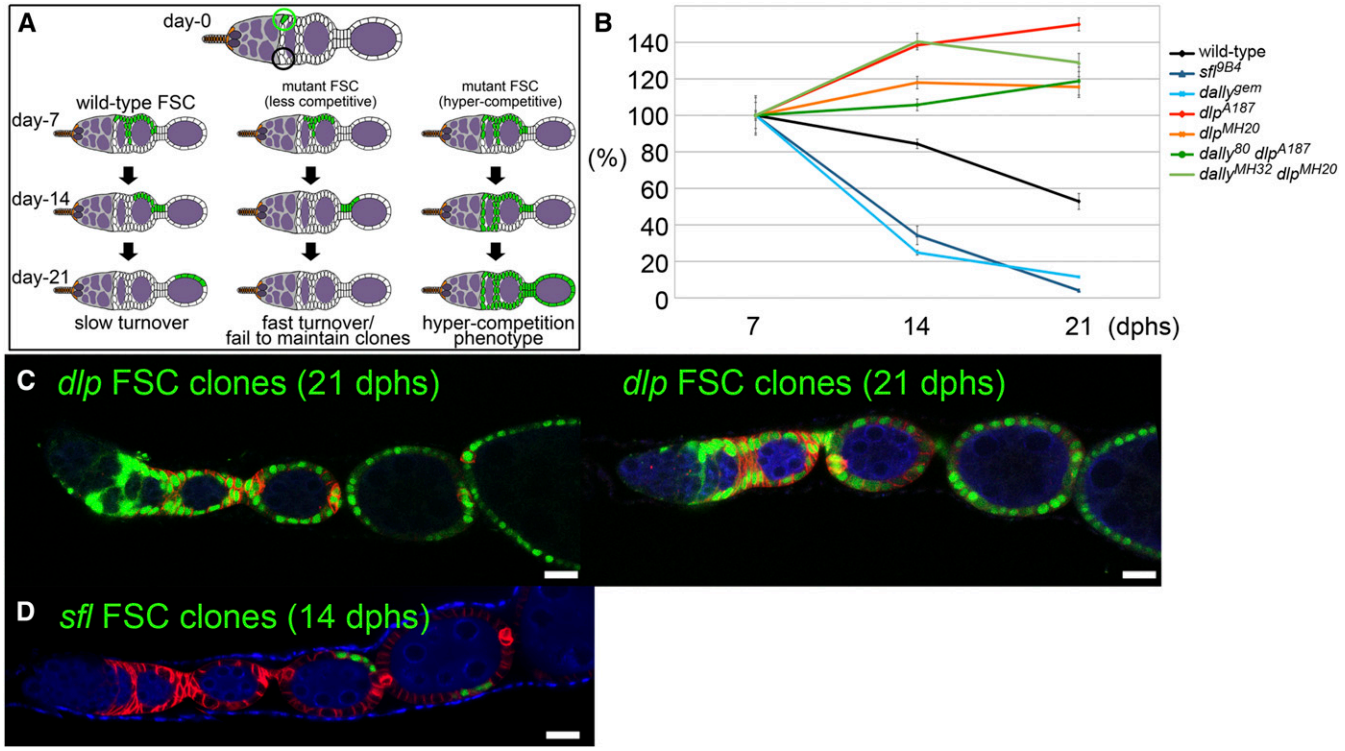

Figure 2 Glypicans are required for normal maintenance and competition for niche occupancy of FSCs. (A) An illustration of FSC lineage analysis using MARCM ("Maintenance Assay"). Green cells represent the GFP-positive mutant (or wild type) FSC lineage. Unmarked cells represent wild-type cells. GFP-positive FSC clones are induced by heat shock, which expresses Flp recombinase. Heat shock conditions are optimized to maximize the number of germaria containing only one GFP-positive FSC. When the GFP-positive FSC is wild type, a slow decrease in marked FSC clone frequency is observed due to normal slow turnover (left). If a GFP-labeled FSC is mutant for a gene required for normal FSC maintenance, the frequency of mutant FSC clones will decrease more rapidly than wild type (middle, less competitive phenotype). In contrast, if a mutation in an FSC causes hyper-competition, the frequency of mutant FSC clones will not decrease (right, hypercompetitive phenotype). Once both niches are occupied by mutant FSCs, the GFP-positive progenitors will eventually cover the entire epithelial sheet (all-marked phenotype). (B) FSC maintenance assay. The percentages of germaria containing GFP-positive FSC clones for the control (black), *sfl*^{9B4} (dark blue), *dally*^{gem} (light blue), *dlp*^{A187} (red), *dlp*^{MH20} (orange), *dally*^{80} *dlp*^{A187} (dark green), and *dally*^{MH32} *dlp*^{MH20} (light green) at 7, 14, and 21 dphs are shown. All values are normalized to the 7 dphs value (100%). In the control, the percentage decreases over time due to natural stem cell turnover. *sfl* and *dally* mutant FSC clones are more quickly lost compared to wild type. *dlp* and *dally dlp* double mutant FSCs persist in the germarium over time. Error bars represent SEM. (C) All-marked phenotype of *dlp* mutant clones. GFP-marked *dlp* mutant cells tend to occupy the entire follicular epithelium, exhibiting the all-marked phenotype. Two examples are shown for ovarioles with all-marked phenotype of *dlp* mutant FSC progenitors (green). Such *dlp* all-marked ovarioles are morphologically normal and do not show follicle cell overproliferation. (D) *sfl* mutant clones are rapidly lost from the germarium, resulting in a germarium with no marked clones. Bar, 10 μm.

Vied and Kalderon 2009; Vied *et al.* 2012). This turnover rate is very similar to previous observations (Song and Xie 2003; Johnston *et al.* 2016).

To determine if HSPGs are involved in FSC maintenance and competition, we first generated *sfl* mutant FSC clones and analyzed the FSC clone frequency. *sfl* encodes the only *Drosophila* HS *N*-deacetylase/*N*-sulfotransferase. *N*-sulfation of glucosamine residues, catalyzed by Sfl, is the first step in a sequence of HS modifications, and is essential for all subsequent modifications of HS chains. Thus, loss of *sfl* disrupts most, if not all, activities of HS chains and in turn impairs all known HS-dependent pathways (Lin and Perrimon 1999; Lin *et al.* 1999). We found that *sfl* null mutation caused a substantial decrease in the frequency of mutant FSCs over time compared to wild-type controls (Figure 2B and Table 1). The *sfl* mutant progenitors disappear more quickly than wild-type FSCs and were almost eliminated from the germarium at 21 dphs (Figure 2B and D). This finding indicates that HSPGs are essential for FSC maintenance.

We next examined the behavior of *dally* mutant FSCs. We found that *dally* mutant FSC progenitors were also rapidly lost from the germarium (Figure 2B). The severity of *dally* mutant phenotype was similar to that of *sfl*, suggesting that Dally is a primary HSPG regulator contributing to normal maintenance and/or competition of FSCs.

On the other hand, unexpectedly, a similar assay using *dlp*^{A187} MARCM clones revealed that *dlp* mutant FSCs behave in the opposite manner to *sfl* and *dally*. The frequency of *dlp* mutant FSCs did not decrease over time, showing that *dlp* mutant FSCs are more likely to remain in the niche compared to wild-type cells (Figure 2B). In many instances, we found that the GFP-marked *dlp* mutant cells occupy the entire follicular epithelium, exhibiting the "all-marked" phenotype (Figure 2C). The all-marked ovarioles covered with *dlp*

Table 1 Summary of FSC maintenance assay

	dphs					
	7		14		21	
GFP-positive clones	−	+	−	+	−	+
Wild type	267	253 (100)	299	235 (84.4)	272	111 (52.9)
sfl^{9B4}	372	117 (100)	548	54 (34.3)	356	4 (4.0)
dally^{gem}	237	20 (100)	305	6 (24.8)	222	2 (11.5)
dlp^{A187}	149	114 (100)	110	165 (138.4)	82	152 (149.9)
dlp^{MH20}	188	111 (100)	258	201 (118.0)	233	175 (115.5)
dally^{80} *dlp*^{A187}	282	165 (100)	329	206 (105.7)	257	189 (118.8)
dally^{MH32} *dlp*^{MH20}	221	105 (100)	178	147 (140.4)	176	125 (128.9)

Numbers of germaria with (+) or without (−) GFP-marked FSC clones of each genotype are shown for 7, 14, and 21 dphs. Values in parentheses in (+) columns show percentages of germaria containing GFP-marked FSC clones at 14 and 21 dphs normalized to that of 7 dphs (100%) in each genotype.

mutant follicle cells are morphologically normal and do not show follicle cell overproliferation. Thus, *dlp* appears to affect FSC replacement without affecting the proliferation of FSCs and progenitor cells.

Since this behavior of *dlp*^{A187} mutant FSCs was surprising, we also used *dlp*^{MH20}, another *dlp* mutant allele generated independently from *dlp*^{A187}. The *dlp*^{MH20} mutant FSC clones were also retained at approximately the same frequency as *dlp*^{A187} over time (Figure 2B). This confirmed that *dlp* mutant FSC behavior is not an allele-specific effect, but indeed reflects *dlp* gene function in terms of FSC niche occupancy. We also observed that *dally-dlp* double mutant FSC clones showed similar behaviors as *dlp* single mutant FSCs (Figure 2B). This result suggests that *dlp* is genetically epistatic to *dally* for this regulation.

Glypicans regulate FSC niche competition

The result of the maintenance assay suggested the possibility that *dlp* is a hypercompetitive mutant, *i.e.*, *dlp* mutants are more competitive for the stem cell niche than wild-type cells. To determine if *dlp* is a hypercompetitive mutant, we examined the effect of mutations on FSC competition by assessing replacement bias. We first measured the proportion of germaria with zero, one, or two marked FSC clones at 7 dphs and monitored the replacement of FSCs after 14 days (at 21 dphs). When the replacement occurs in a germarium with one marked FSC clone, there are two possible fates: either the marked FSC is replaced by an unmarked FSC daughter, resulting in a germarium with no marked clone, or the marked FSC daughter replaces the unmarked FSC, producing a germarium with two marked FSC clones (Figure 3A). The ratio of these two events reflects the competitiveness of the marked FSC for niche occupancy. When the GFP-marked FSC is wild type, it does not show any bias for the two events (Figure 3B and Table 2). Both *sfl* and *dally* mutant FSCs are fully biased toward zero marked clones, indicating that they are much less competitive. On the other hand, *dlp* mutant as well as *dlp-dally* double mutant FSCs are indeed hypercompetitive. Thus, the glypicans affect FSC competitiveness, directly altering the replacement bias, rather than other processes that could indirectly affect FSC maintenance, such as cell proliferation and apoptosis.

Roles of glypicans in Jak/Stat signaling in FSCs

Previous studies have identified several pathways that affect FSC maintenance, including Jak/Stat, Wg, and Hh signaling (Zhang and Kalderon 2000; Song and Xie 2003; Vied *et al.* 2012; Sahai-Hernandez and Nystul 2013). The ligands of these pathways, Upd, Wg, and Hh, are well established as HS-dependent factors (Hayashi *et al.* 2012; Nakato and Li 2016). To understand the molecular mechanisms by which glypicans control stem cell competition, we systematically analyzed the effects on these three pathways of RNAi knockdown of the glypicans in the germarium.

A recent study proposed that the Jak/Stat and Hh signaling pathways are the major regulators of FSC behavior (Vied *et al.* 2012). We first examined whether *dally* and *dlp* play roles in regulating Jak/Stat signaling in the germarium. We expressed UAS-RNAi transgenes by *109-30-Gal4*, a commonly used follicle cell driver (Vied *et al.* 2012). GFP expression using this driver confirmed that it is strongly expressed in the follicle cell populations, including FSCs and prefollicle cells (Figure 4A). The *10xSTAT-GFP* reporter was used as a readout for Jak/Stat signaling (Bach *et al.* 2007; Hartman *et al.* 2015). GFP reporter expression shows a gradient of Jak/Stat signaling with the highest levels near polar cells, the source of the Upd ligand (Figure 4, B and B'). We found that *sfl* RNAi knockdown by the *109-30-Gal4* driver dramatically decreased the *10xSTAT-GFP* signal, disrupting the Jak/Stat gradient (Figure 4, C and C'). This is often accompanied with reduced length or loss of the stalks and a fused egg chamber phenotype (discussed in the section "Glypicans regulate stalk cell differentiation"). High-magnification images of the germaria revealed that *sfl* RNAi also reduces the *10xSTAT-GFP* reporter signal in the area of FSCs (Figure 4, D–E'). Quantification of GFP signal intensity specifically over the area of FSCs confirmed the reduction of Jak/Stat signaling in FSCs by *sfl* RNAi (Figure 4F). Similar assays revealed that *dally* RNAi knockdown also resulted in impaired Jak/Stat signaling in FSCs (Figure 4F) and loss of stalks (Figure 5B). This result is consistent with our previous finding that Dally serves as a coreceptor for Upd (Hayashi *et al.* 2012). This reduction of Jak/Stat signaling could explain why *sfl* and *dally* mutant FSCs are less competitive for niche occupancy. We found that *dlp* RNAi also strongly impaired Jak/Stat signaling in FSCs monitored by *10xSTAT-GFP* (Figure 4F). These results indicate that both glypicans serve as coreceptors for Upd, acting as positive regulators of Jak/Stat signaling.

Glypicans regulate stalk cell differentiation

As mentioned above, RNAi knockdown of *sfl* and *dally* by the *109-30-Gal4* driver resulted in fused egg chambers lacking the stalk structure (Figure 5, A and B). This is likely caused by the disruption of Jak/Stat signaling, which plays a major role in stalk cell differentiation (Baksa *et al.* 2002; McGregor *et al.* 2002; Hayashi *et al.* 2012). To determine the relative contributions of the glypicans to stalk development, we quantified

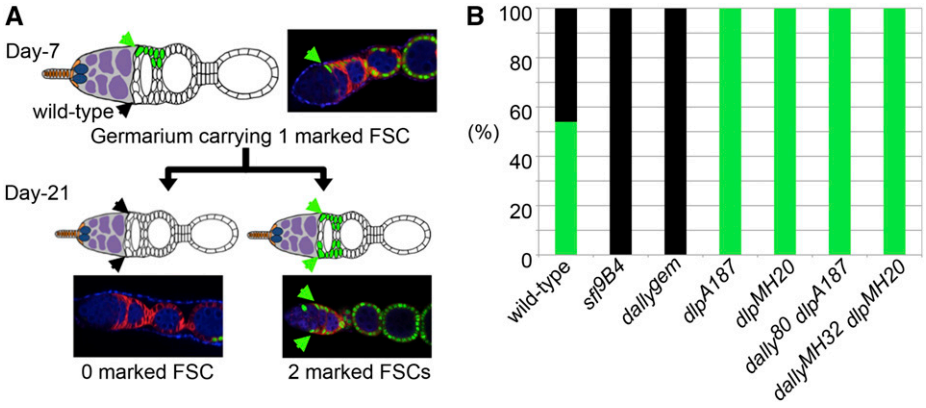

Figure 3 FSC replacement bias assay. Glypicans regulate competition for the niche occupancy of FSCs. (A) An illustration of the FSC replacement bias assay. In a germarium with one marked FSC clone, there are two possible fates for the marked FSC: either the marked FSC is replaced by an unmarked FSC daughter resulting in germarium with no marked clone, or the marked FSC daughter replaces the unmarked FSC, producing two marked FSC clones. The ratio of these two events reflects the competitiveness of the marked FSC for niche occupancy. Arrowheads mark the FSCs. (B) The ratio of the replacement, which caused an increase of germaria containing zero marked clones (black) or two marked clones (green), between 7 and 21 dphs is shown for each genotype.

the effects of RNAi knockdown of *sfl*, *dally*, and *dlp* on stalk cell number in the first and second stalks.

RNAi knockdown of *sfl* and *dally* in follicle cells led to the complete loss of stalks in most ovarioles: the median value of stalk cell number was zero in these genotypes (Figure 5C). Although the phenotype was not as severe as *sfl* and *dally* RNAi, the reduction of stalk cells in *dlp* knockdown ovarioles was also statistically significant. This finding suggests that both Dally and Dlp serve as potent Upd coreceptors during early oogenesis. At later stages, Dlp plays a minor role in this pathway when its expression shifts from somatic to germline cells (Figure 1, D and D').

Roles of glypicans in Wg signaling in FSCs

Wg has been suggested as a candidate niche factor derived from the escort cells and Wg signaling is critical for FSC maintenance (Song and Xie 2003; Sahai-Hernandez and Nystul 2013). Therefore, we asked if the glypicans affect Wg signaling in FSCs using *fz3-RFP*, a Wg signaling reporter (Olson *et al.* 2011; Wang and Page-McCaw 2014). In control, *fz3-RFP* expression is detected in the escort cells as well as FSCs (Figure 6A). The signal decreases in differentiating follicle cells that are located posterior to FSCs. When the *sfl* RNAi transgene was expressed by *109-30-Gal4*, the signal intensity of *fz3-RFP* was significantly reduced in FSCs and more posteriorly located cells where *109-30-Gal4* is expressed (Figure 6B). The signals in more anterior cells were unaffected. As a result, the strong *fz3-RFP* signal intensity sharply drops near the 2a/b border (arrows in Figure 4B').

Quantification of RFP signal intensity over the area of FSCs revealed that Wg signaling in FSCs was severely disrupted by RNAi knockdown of *sfl* and *dlp* (Figure 6C). This effect of *dlp* RNAi in Wg signaling is consistent with a previous observation (Wang and Page-McCaw 2014). Interestingly, *dally* RNAi did not show a significant effect on *fz3-RFP* signal intensity in FSCs.

It is well established that Wnt signaling is controlled by another class of cell surface HSPGs, Sdcs, in many cellular and developmental contexts (Alexander *et al.* 2000; Astudillo *et al.* 2014; Dejima *et al.* 2014; Pataki *et al.* 2015; Saied-Santiago *et al.* 2017). We found that *Drosophila* Sdc is expressed in both somatic and germline cells in the germarium, including FSCs (Figure S1A in File S1). Therefore, we examined the effect of *Sdc* RNAi knockdown on Wg signaling in FSCs. As shown in Figure S1B in File S1, Wg signaling was significantly compromised by *Sdc* RNAi, showing that Sdc also regulates this pathway, in addition to Dlp. Taken together, our results show that Dlp and Sdc act as primary Wg coreceptors in FSCs.

Roles of glypicans in Hh signaling in FSCs

Another pathway essential to normal FSC maintenance is Hh signaling (Forbes *et al.* 1996; Zhang and Kalderon 2000; Hartman *et al.* 2010; Vied *et al.* 2012). We monitored Hh signaling activity in the germarium using a *ptc-GFP* reporter (Ulmschneider *et al.* 2016). Expression of the *sfl* RNAi construct in follicle cells using the *109-30-Gal4* driver reduced Hh signaling in FSCs (Figure 7, A–B'). However, the effects of individual knockdown of either *dally* or *dlp* alone were modest, and our assay did not show a statistically significant difference (Figure 7C). To examine the genetic redundancy between these glypican molecules, we analyzed the effect of *dally dlp* double RNAi knockdown on Hh signaling. Simultaneous expression of *dally* and *dlp* RNAi constructs in FSCs significantly reduced Hh signaling (Figure 7C). In contrast, no significant effect was observed by *Sdc* RNAi (Figure S1C in File S1). Our results suggest that the two glypicans redundantly function to enhance Hh signaling.

Taken together, our study shows that HS biosynthesis and glypicans are required for FSC maintenance and the regulation of niche competition behavior. Dally and Dlp play both partially redundant and specific roles in regulating multiple signaling pathways in FSCs. The fact that glypicans regulate a large number of HS-dependent factors suggest roles of Dally and Dlp in orchestration and modulation of signaling dosage of different pathways to control FSC maintenance and replacement.

Table 2 Summary of FSC replacement bias assay

	dphs								
	7			14			21		
GFP-positive FSCs	0	1	2	0	1	2	0	1	2
Wild type	60.5	26.8	12.7	56.5	22.4	21.1	66.7	13.4	20
sfl^{9B4}	83.1	16.0	1.0	93.6	6.4	0	98.1	1.9	0
dally^{gem}	92.2	7.8	0	98.1	1.9	0	99.1	0.9	0
dlp^{A187}	56.7	23.2	20.2	40	19.6	40.4	35.0	23.9	41.0
dlp^{MH20}	62.9	30.4	6.7	56.0	27.4	16.6	57.5	22.1	20.4
dally^{80}*dlp*^{A187}	62.6	22.6	14.8	60.6	18.1	21.3	55.5	22.3	22.3
dally^{MH32}*dlp*^{MH20}	67.8	19.0	13.2	54.8	23.7	21.5	58.5	18.3	23.3

Percentages of germaria containing 0, 1, or 2 marked FSC clones at 7, 14, and 21 dphs.

Discussion

During adult life, stem cells are often replaced by progenitor cells through niche competition to maintain stem cell quality (Nystul and Spradling 2007, 2010). Regular competition between stem cells for niche occupancy can result in two major consequences. First, unhealthy or old stem cells can be eliminated from the niche, and thus the niche is always inhabited with a healthy stem cell population for tissue homeostasis and regeneration. Second, the stem cell could also potentially acquire spontaneous mutations that enhance stem cell competitiveness for niche occupancy. If a mutation in a FSC causes hyper-competition, these mutant progenitors often occupy the entire epithelial sheet eventually (all-marked phenotype). This phenomenon resembles an early phase of cancer formation, in which a stem cell accumulates spontaneous, competitive mutations, and mutant progenitors aggressively expand over the tissue (Visvader 2011). It has been proposed that human precancerous mutations spread from niche to niche in a similar manner, by stem cell competition, increasing their representation within a tissue field (Nystul and Spradling 2007; Shiozawa *et al.* 2011). Thus, the FSC niche offers an excellent model for epithelial cancers.

Our study shows that *dally* mutant FSCs are less competitive for niche occupancy compared to wild type, while *dlp* mutant FSCs show hypercompetitive behavior. Thus, the two *Drosophila* glypicans act in an opposing fashion in FSC competition. These results are consistent with previous observations that some human glypicans are oncogenic (similar to *dally* in the *Drosophila* ovary) and others are tumor suppressors (similar to *dlp*) (Pilia *et al.* 1996; Cano-Gauci *et al.* 1999; Li *et al.* 2004; Williamson *et al.* 2007). Our maintenance and replacement bias assays showed that *sfl* FSC mutant phenotype was similar to *dally*. We also observed that *dally dlp* double mutant FSCs behave in a similar manner to *dlp*. One possible explanation for the difference between *sfl* and *dally dlp* double mutant phenotypes is that other HSPG molecules are also involved in regulating FSC behavior. Since Sdc affects Wg signaling in FSCs (Figure S1 in File S1), it is likely that Sdc participates in this control. It is also possible that Dlp may have some functions that are not entirely dependent on HS. Previous studies showed that mutated HSPG core-proteins with no HS chain retain the ability to rescue mutant phenotypes caused by loss of respective HSPG molecules (Kirkpatrick

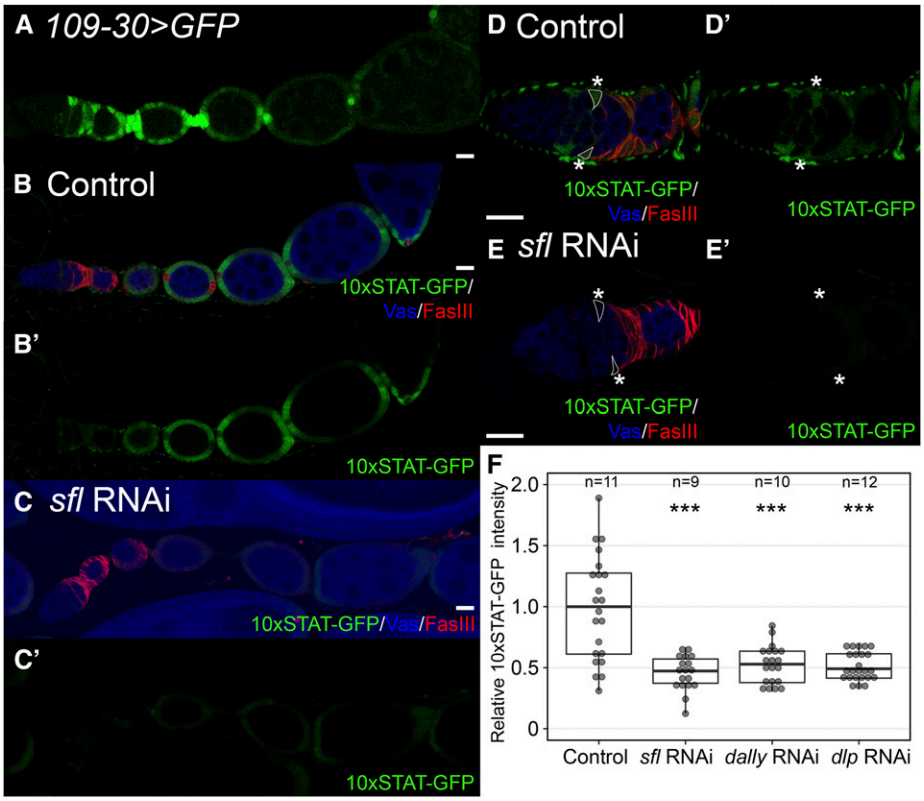

Figure 4 Roles of glypicans in Jak/Stat signaling in FSCs. (A) Expression patterns of *109-30-Gal4*. GFP signal in a *109-30>GFP* ovary shows a high level of expression in the follicle cell populations, including FSCs. (B–E') *10xSTAT-GFP* reporter assay in control (B, B', D, and D') and *109-30>sfl* RNAi (C, C', E, and E') ovaries. Low magnification images (B–C') show that *sfl* RNAi dramatically reduced GFP signal intensity (green) in the follicle cells. High magnification views (D–E') reveal that GFP signal intensity over the FSC areas is significantly decreased by *sfl* RNAi. Ovarioles are stained for Vas (blue) and *Fas*III (red). White outlines and asterisks mark FSC areas. (F) Quantification of Jak/Stat signaling in FSCs. GFP signal intensity was measured over the area of FSCs of indicated genotypes. The intensity values in control samples were set to 1.0, and relative intensities in RNAi knockdown FSCs were calculated. Numerical figures depict the mean ± SE. *** $P < 0.001$ (Student's *t*-test). Each dot represents an individual FSC examined. n, number of germaria assayed. Bar, 10 μm.

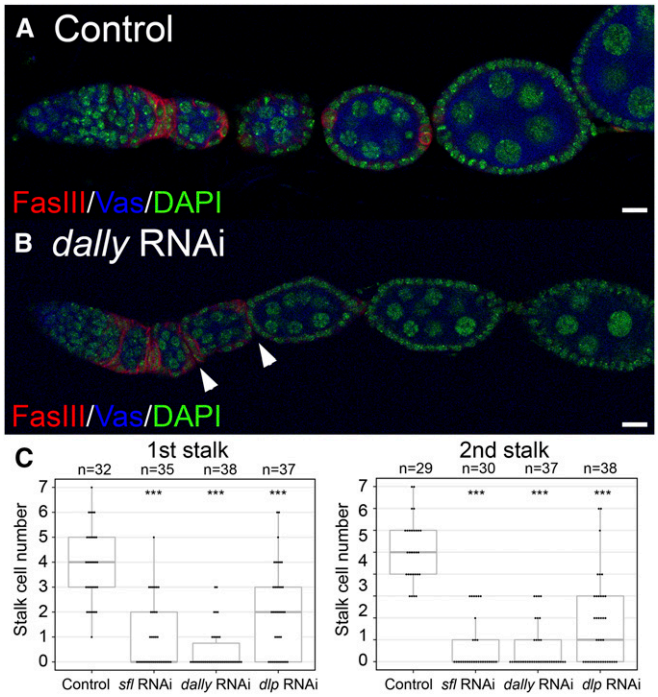

Figure 5 Glypicans regulate stalk cell development. (A and B) Ovarioles of control (*109-30-Gal4*; A) and *109-30>dally* RNAi (B) stained for DAPI (green), Vas (blue), and *Fas*III (red). Arrowheads mark loss of stalks. (C) Quantification of stalk cell numbers (mean ± SE). Results are shown for the first (left) and second (right) stalks. Significance values are relative to control. *** $P < 0.001$ (Mann–Whitney–Wilcoxon test). n, number of ovarioles assayed. Bar, 10 μm.

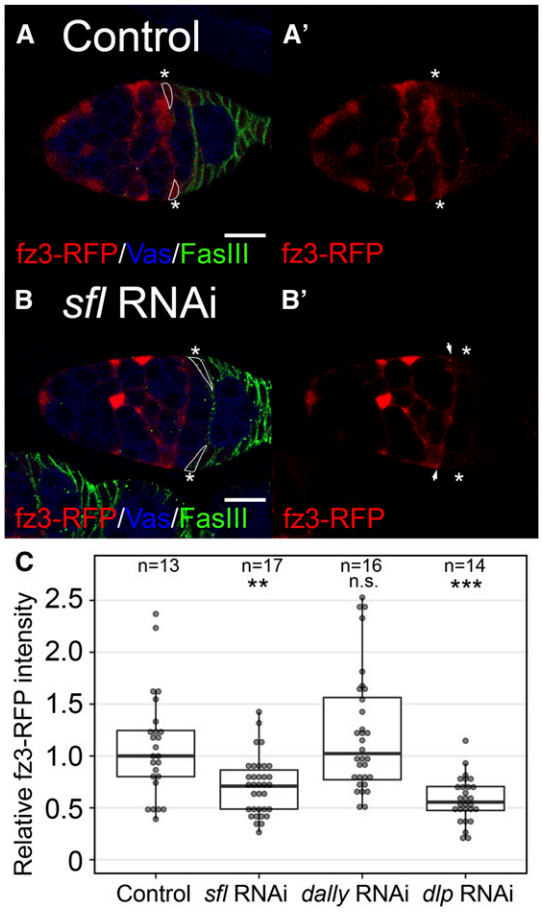

Figure 6 Roles of glypicans in Wg signaling in FSCs. (A–B') *fz3-RFP* reporter assay in control (A and A') and *109-30>sfl* RNAi (B and B') ovaries. RFP signal intensity (red) is significantly reduced over the area of FSCs by *sfl* RNAi. Note that RFP signal in the anterior escort cells in which *109-30-Gal4* is not expressed is not affected. Ovarioles are stained for Vas (blue) and *Fas*III (green). White outlines and asterisks mark FSC areas. (C) Quantification of Wg signaling in FSCs. fz3-RFP signal intensity was measured over the area of FSCs of indicated genotypes. The intensity values in control samples were set to 1.0, and relative intensities in knockdown FSCs were calculated. Numerical figures depict the mean ± SE. ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test). Each dot represents an individual FSC examined. n, number of germaria assayed. Bar, 10 μm.

et al. 2006; Yan *et al.* 2009; Dejima *et al.* 2014; Saied-Santiago *et al.* 2017).

We found that a reduction of *dally* in FSCs had a major effect on Jak/Stat signaling. The disruption of the Jak/Stat pathway can account for the compromised FSC maintenance and competition of *dally* mutant stem cells (Vied *et al.* 2012). On the other hand, further study is required to elucidate the molecular basis for *dlp*'s function in FSC control. We found that *dlp* mutant FSCs show a hypercompetitive behavior but we did not identify any growth factor pathways that are negatively controlled by Dlp. One interesting aspect of *dlp* phenotype is that although *dlp* mutant progenitors occupy both niches and show an all-marked phenotype, the overall morphology of such mutant ovarioles is normal. This shows a striking contrast with mutations in known negative regulators of the Wg (Song and Xie 2003) and Hh (Zhang and Kalderon 2000; Hartman *et al.* 2010) pathways. All these mutant ovarioles show, in addition to the all-marked phenotype, overproliferation of follicle cells, leading to gross morphological defects of ovarioles. This difference between *dlp* and known negative regulators of the growth factor pathways suggest the possibility that *dlp* regulates an HS-dependent, nongrowth promoting factor. One such candidate pathways is Slit-Robo signaling. Slit is a HS-dependent factor and requires HSPGs on the target cell membrane (Inatani *et al.* 2003; Hohenester 2008). Although Slits are well established as an axon guidance cue, recent studies have revealed that Slit and Robo receptors regulate many developmental processes by influencing cell polarity and adhesion in non-neuronal tissues (Ypsilanti *et al.* 2010). In *Drosophila*, Slit-Robo signaling plays critical roles in the male GSC niche in the testis (Stine *et al.* 2014), intestinal stem cells in the midgut (Biteau and Jasper 2014), and tracheal system (Schulz *et al.* 2011). In fact, Slit is known to be expressed in the FSC niche (Nystul and Spradling 2010; Reich and Papoulas 2012) and we found that Robo receptors are also present in this region (Figure S2 in File S1). Thus, Dlp may show an opposing activity to Dally by controlling additional pathway(s), such as Slit-Robo signaling, rather than competing with Dally to downregulate Dally-dependent growth factor signaling.

Our current model is depicted in Figure 8. HS-dependent FSC niche factors and signaling proteins are released from

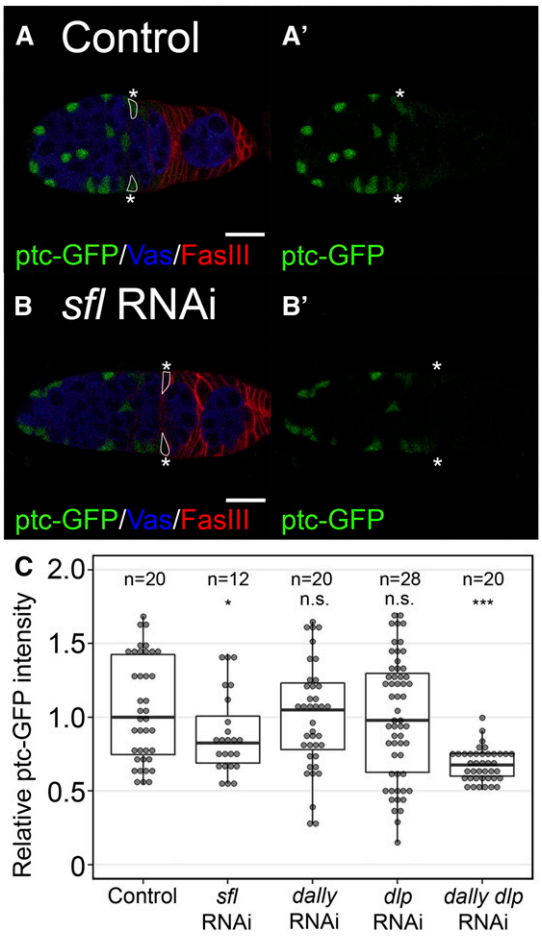

Figure 7 Roles of glypicans in Hh signaling in FSCs. (A–B') *ptc-GFP* reporter assay in control (A and A') and *109-30>sfl* RNAi (B and B') ovaries. White outlines and asterisks mark FSC areas. GFP signal, Vas, and *Fas*III are shown in green, blue, and red, respectively. (C) Quantification of Hh signaling in FSCs (mean ± SE). ptc-GFP signal intensity was measured over the area of FSCs of indicated genotypes. The intensity values in control samples were set to 1.0, and relative intensities in knockdown FSCs were calculated. * $P < 0.05$, *** $P < 0.001$, n.s. not significant (Student's *t*-test). Each dot represents an individual FSC examined. n, number of germaria assayed. Bar, 10 μm.

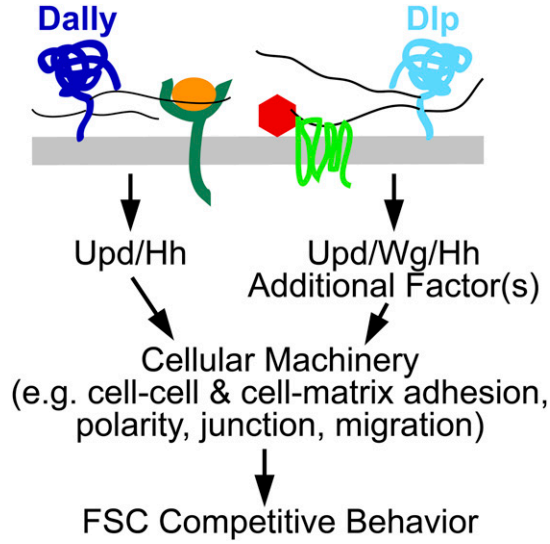

Figure 8 Model for regulation of FSC competition by glypicans. Glypicans regulate signaling dosage of multiple signaling pathways mediated by HS-dependent factors, including Upd, Wg, and Hh, on the surface of FSCs. Dlp is likely to regulate additional factor(s). Such signaling could regulate cellular machineries, such as those involved in cell adhesion, FSC daughter cell migration, and cell polarity, which directly affect the competitive behavior of FSCs. Thus, different inputs from Dally and Dlp are combined together to achieve normal (wild type) competitive behavior.

surrounding cells such as posterior escort cells or polar cells posterior to the niche (Vied *et al.* 2012; Sahai-Hernandez and Nystul 2013). When these ligand molecules activate their respective receptors on the FSC surface, they also bind to Dlp and/or Dally coreceptors, which modulate signaling dosage. This consequently controls downstream cellular machineries, such as those involved in cell adhesion, FSC daughter cell migration, and cell polarity, which affect FSC competitive behavior. Such HS-dependent factors include Upd, Wg, and Hh, but unidentified molecules are also likely to be involved. It has been proposed that FSC behavior is controlled by the sum of inputs from multiple signaling pathways (Vied *et al.* 2012). Since both glypicans regulate a number of signaling, differential behaviors of *dally* and *dlp* mutant FSCs may reflect combined effects or the interactions of multiple pathways that are altered differently in each mutant FSC. For example, since we found that the effects of *dally* and *dlp* mutations on Wg signaling are different, the interaction between Wg and other pathways (*e.g.*, Jak/Stat signaling) might contribute to their phenotypes. Our results suggest that the glypicans combine and orchestrate multiple signaling pathways to regulate FSC behaviors.

Although it is clear that HSPGs regulate signaling events mediated by a number of HS-dependent factors, how HSPGs exert specific functions remains a central question in proteoglycan biology. GPC3, one of six mammalian glypicans, binds to Sonic hedgehog and competes with Patched for Hh binding (Capurro *et al.* 2008, 2009). GPC3 induces the endocytosis and degradation of Hh, thus acting as a negative regulator of the pathway. Loss-of-function mutations in GPC3 were identified as the cause of a rare X-linked condition characterized by pre- and postnatal overgrowth (Pilia *et al.* 1996). It is believed that enhanced Hh signaling caused by loss of GPC3 contributes to the developmental abnormalities of the disease. On the other hand, GPC5, another member of the glypican family, increases the binding of Sonic hedgehog to Patched, acting as a positive regulator (Li *et al.* 2011). GPC5 was found to be significantly upregulated in a soft tissue sarcoma, rhabdomyosarcoma (Williamson *et al.* 2007). In addition, a recent study demonstrated that GPC6, the loss of which causes autosomal-recessive omodysplasia, also stimulates Hh signaling (Capurro *et al.* 2017). Thus, glypican coreceptors can act as an either stimulatory or inhibitory regulator of Hh signaling. Opposing activities of Dally and Dlp in FSC competition also highlight the diversity and specificity of glypican functions.

Glypican molecules can be classified into two major groups based on structural similarities and the activities on Hh signaling: Dlp is similar to GPCs 1, 4, and 6, whereas Dally appears to be close to GPCs 3 and 5 (Williams *et al.* 2010). We do not observe a direct correlation between the functions of *dally* and *dlp* in FSC control and the oncogenic and tumor suppressor activities of mammalian glypicans from the respective groups, although both Dally and GPC5 act as a positive coreceptor of Hh. Further studies will be required to elucidate the molecular mechanisms by each glypican exerts specific functions to differentially affect signaling and patterning. The fine structure of HS chains has a major effect on HSPG function. It is interesting to know how mutations in HS modifying enzyme genes affect FSC maintenance and competition.

Acknowledgments

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